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Deuterons cannot replace protons in active transport processes in yeast

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Replacement of ordinary water with heavy water causes a sharp reduction of the rates of both primary hydrogen ion transport (at the plasma membrane ATPase) and secondary symports (H^+-associated transports of sugars and amino acids) in several species of yeast. At the same time, the hydrolytic activity of the ATPase is affected only very little. Likewise, the membrane potential, the ΔpH and, correspondingly, the accumulation ratios of the various symported solutes are altered much less. This serves as evidence that H^+ or H_3O^+ ions are direct participants in the various active transports of nutrients in yeast.

Heavy water; Plasma membrane; Proton symport; H^+-ATPase; Yeast

1. INTRODUCTION

A large amount of data on the involvement of protons in various solute transports in yeast has accumulated over the past 15 years (see [1–4] as examples) even if the evidence was often unconvincing [5] and unsatisfactory in quantitative terms [6]. It could even be argued that the phenomena observed on adding a presumably H^+-symported solute to a yeast suspension, such as transient alkalization of the outside medium or depolarization of the membrane, could be explained by assuming the hydrolysis of ATP to be the source of energy for both the proton-extruding ATPase and for the solute transports.

It appeared that an unequivocal proof for the involvement of protons could be obtained by using a potential competitor, such as the deuteron or, if hydronium ions H_3O^+ are the functional ionic species, D_3O^+. The results presented here do in fact provide such proof.

2. MATERIALS AND METHODS

Three yeast species were used here: *Saccharomyces cerevisiae* K. (CCY 21-4-60), *Rhodotorula gracilis* (CCY 20-2-16; ATCC 26194), and *Lodderomyces elongisporus* (CCY 65-1-1; ATCC 11503). All these species display a variety of secondary active transports, with the notable exception of *S. cerevisiae* which transports monosaccharides, including its major substrate D-glucose, by mediated diffusion [7].

The cells were grown aerobically at 30°C for 21 h (*S. cerevisiae*), 24 h (*R. glutinis*) and 24 h (*L. elongisporus*) to reach the early stationary phase in a glucose/mineral salts medium described before [8]. The cells were harvested, washed and aerated for 2 h to deplete their endogenous reserves. Prior to transport experiment, *S. cerevisiae* was preincubated for 1 h with 1% D-glucose to start synthesis of transport proteins [9]. Only then were the cells washed and resuspended in heavy water.

Transport of labelled solutes was estimated as in [9], intracellular pH as described by Slavík [10] and transmembrane electrical potential with tetraphenylphosphonium ions [11].

The extracellular pH of suspensions was monitored with a combination glass electrode from Radiometer (Denmark) on a flatbed recorder (Laboratorní přístroje, Czechoslovakia).

The ATP-hydrolyzing activity was assayed in membrane fragments as described by Serrano [12] with the modification that 20 mM KNO_3 was added to the assay mixture to block any vacuolar ATPase that might be present in the preparation [13].

Heavy water (99%, with tritium admixture amounting to less than 100 kBq/l) was obtained from the Institute for Nuclear Research (Czechoslovakia).

The tagged sugars and amino acids were all uniformly labelled and were obtained from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia), with the exception of tritiated 6-deoxy-D-glucose which was prepared in this laboratory some time ago. ^{14}C-Labelled tetraphenylphosphonium was from Amersham International (UK). All the nonlabelled chemicals were either from Koch-Light Genzyme (UK) or from Lachema (Czechoslovakia) and were of the highest purity available.

3. RESULTS

The effect of heavy water on facilitated diffusion was studied on the model of 6-deoxy-D-glucose uptake by baker's yeast and was found to be virtually nil (Table I) which, among other things, indicates that no significant alteration of the carrier structure and conformation of its specific binding site for monosaccharides took place in the presence of D_2O.

On the other hand, the uptake of amino acids by *S. cerevisiae*, as well as that of 6-deoxy-D-glucose by *R. gracilis* and *L. elongisporus*, all of these being believed to proceed by proton symport, were profoundly decreased in the presence of D_2O (Fig. 1, Table I). The effect of heavy water was apparently mainly on the

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Table I

Effects of D_2O on membrane associated processes in yeast[a]

Type of process	R
Transport of 0.1 mM 6-deoxy-D-glucose in *Saccharomyces cerevisiae*	0.94 ± 0.12
Transport of 0.1 mM 6-deoxy-D-glucose in *Lodderomyces elongisporus*	0.61 ± 0.15
Transport of 10 mM 6-deoxy-D-glucose in *Lodderomyces elongisporus*	0.38 ± 0.08
Transport of 0.1 mM L-tryptophan in *Saccharomyces cerevisiae*	0.31 ± 0.13
H^+ extrusion by plasma membrane ATPase in starved cells of *S. cerevisiae*	0.09 ± 0.03
H^+ extrusion by plasma membrane ATPase in glucose-pretreated cells of *S. cerevisiae*	0.26 ± 0.13
ATP hydrolysis by plasma membrane ATPase from starved cells of *S. cerevisiae*	0.96 ± 0.07
ATP hydrolysis by plasma membrane ATPase from glucose-pretreated *S. cerevisiae*	0.97 ± 0.07

[a] R is the ratio of initial rate in D_2O to that in H_2O, and is shown as average from 5–8 experiments ± SD

maximum rate of transport rather than on the half-saturation constant of the given solute. This was demonstrated quantitatively with 6-deoxy-D-glucose in *L. elongisporus* where the half-saturation constant (expressed in mmol/l) was 0.51 in H_2O and 0.44 in D_2O while the maximum rate (expressed in nmol/min/mg dry wt.) was 43.4 in H_2O but 21.0 in D_2O.

Still, the accumulation ratio of that sugar (intracellular divided by extracellular concentration) at 0.1 mM and pH 5.5 in heavy water was 81 ± 11% of that in ordinary water (a mean of 7 experiments), hence hardly a significant depression. This was in fact in qualitative agreement with the simultaneously estimated $\Delta\tilde{\mu}_{H^+}$ (difference in the electrochemical potential of protons) which was found to be 16.0 kJ/mol in H_2O and 16.1 kJ/mol in D_2O (at pH 5.5) in *S. cerevisiae*, 13.4 vs 13.1 in *L. elongisporus* and 17.8 vs 17.1 in *R. gracilis* (all these are means of 6 estimations with SDs not exceeding ± 12% of the mean).

As with the secondary active transports discussed above, the primary active extrusion of H^+ ions through the plasma membrane ATPase [14] was markedly affected by replacing ordinary water with D_2O, the ratio of rates of diethylstilbestrol-sensitive acidification of the outside medium in heavy water to that in ordinary water being shown in Table I.

This contrasts with estimations of ATP hydrolysis by the same enzyme in H_2O and D_2O, there being little difference between the two. ATPase preparations from cells incubated without substrate liberated 68 ± 11 nmol inorganic phosphate/min/mg protein in H_2O and 63 ± 11 nmol in D_2O; preparations from cells incubated with glucose (cf. [15]) yielded 180 nmol phosphate in H_2O and 173 nmol in D_2O. There was an additional release of 45–70 nmol phosphate that was diethylstilbestrol-insensitive and that was equally unaffected by heavy water.

4. DISCUSSION

Of the numerous reports on the use of heavy water in molecular cell research only a few are relevant in the

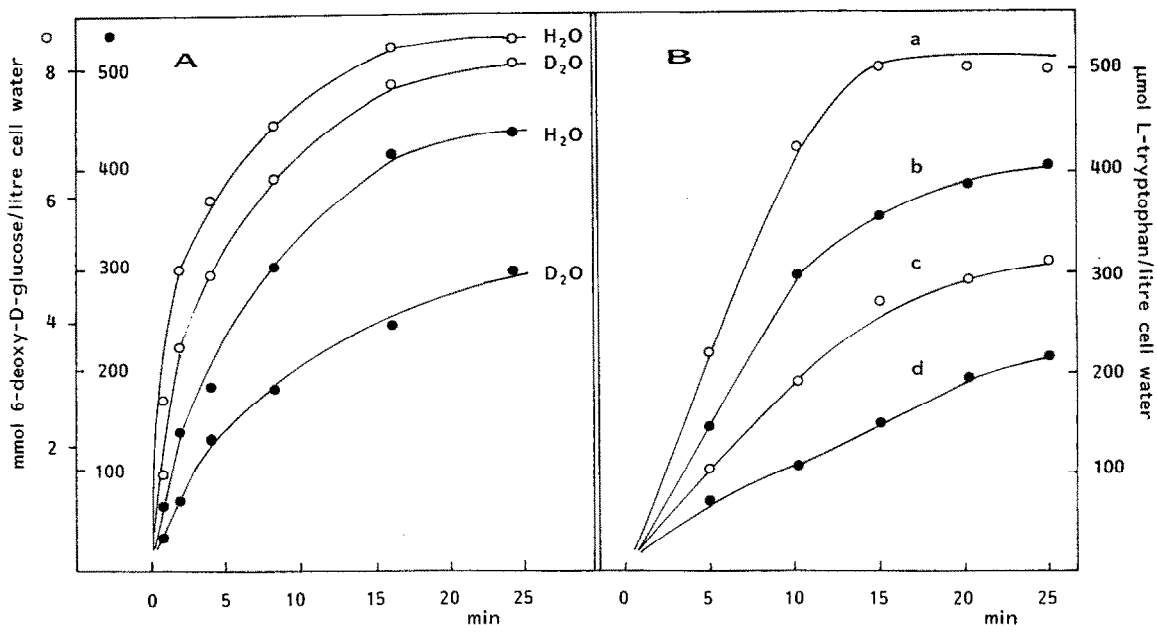

Fig. 1. (A) Uptake of 0.11 mM (blank circles) and 11 mM (solid circles) 6-deoxy-D-glucose by *Lodderomyces elongisporus* aerobically at 30°C in ordinary and heavy water. (B) Uptake of 0.01 mM L-tryptophan by *Saccharomyces cerevisiae* aerobically at 30°C: (a) ordinary water; (b) $H_2O:D_2O = 1:1$; (c) $H_2O:D_2O = 1:9$; (d) heavy water.

present context. Two general observations have been made: (1) a slowing down of molecular as well as cellular reactions due to heavy water (e.g. [16,17]); and (2) stabilization of enzymes and supramolecular structures by heavy water, apparently due to increased hydrophobic interactions in D_2O (e.g. [17–19]). In this paper, the first data on the direct involvement of protons (or deuterons) in transport processes are reported, with the uniform result that replacement of water with D_2O results in a substantial decrease in the rates of the transport involved. This is most marked in the case of the plasma membrane ATPase where the extrusion of H^+ ions is reduced by as much as 90%. The ATP hydrolysis measured in an isotropic system was unaffected by D_2O; compare, in this context, the lack of D_2O effect on the ATP/P_i exchange reaction of the F_0F_1-ATPase [20]. It thus appears that the binding site for protons (or hydronium ions [21]) to be transported does not accept deuterons (or deuteronium ions) with equal ease or perhaps not at all. The fact (not reported here explicity) that in other yeast species the H^+-extruding ATPase activity is only partly suppressed by D_2O suggests that this is rather a quantitative effect than an absolute misfit of D^+ or D_3O^+ for the binding site.

It is matter for discussion whether the graded effects of D_2O on secondary transports are due to direct competition of D^+ (D_3O^+) with H^+ (H_3O^+) or whether they are secondary to the effect on the plasma membrane ATPase. The arguments for the primary role of the ATPase are the following.

(i) The effect of D_2O is much greater on the ATPase than on the secondary transports.

(ii) It is more pronounced at higher concentrations of the transported substrate where the ATPase has to cope with a greater influx of symported H^+ or D^+.

(iii) The effect of D_2O is smaller at low pH values than at high ones (25–42% at pH 3.5 and 47–68% at pH 7). At low pH the secondary transports use mainly the H^+ or D^+ from the external medium, at high pH the ATPase must produce them into the perimembrane space. If this process is blocked by the presence of D_2O, the effect is felt by the secondary transports more than at low pH. Since the pK_a of weak acids is decreased by D_2O it might be argued that the ease with which H^+ (or D^+) dissociates intracellularly from the carrier would be greater in D_2O than in H_2O and thus affect the apparent acid-extruding activity. However, this would in fact reduce the work to be done by the ATPase and hence cannot account for the observation.

On the other hand, the unequal response of various secondary transports to D_2O might be an argument for effects of D^+ directly at the proton-binding site of the secondary transports. It is quite probable that both types of effect are involved in parallel.

As a general conclusion, it appears to be demonstrated that H^+ or H_3O^+ ions are direct participants in the secondary 'proton symports' that abound in yeast and in nature as a whole.

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